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Diminished Intracellular Invariant Chain Expression Following Vaccinia Virus Infection

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Abstract

Vaccinia virus (VV) has been used as a vaccine to eradicate smallpox and as a vaccine for HIV and tumors. However, the immunoevasive properties of VV, have raised safety concerns. VV infection of APC perturbs MHC class II-mediated Ag presentation. Exposure of human B cell lines to VV induced a dramatic reduction in cellular expression of the class II chaperone, invariant chain (Ii) during the late stages (i.e. 8–10 h) of infection. Yet, cell viability and surface expression of MHC class II molecules were maintained up to 24 h after exposure to virus. Reductions in Ii and class II mRNA levels were detected as early as 6 h after VV infection of APC. To examine whether VV was acting solely to disrupt host protein synthesis, B cells were treated with an inhibitor of translation, cycloheximide (CHX). Within 1 h of B cell CHX treatment, Ii protein expression decreased coupled with a loss of class II presentation. Analysis of Ii degradation in VV or CHX treated cells, revealed on-going Ii proteolysis contributing to reduced steady state Ii levels in these APC. Yet in contrast with CHX, VV infection of APC altered lysosomal protease expression and Ii degradation. Virus infection induced cellular cathepsin L expression while reducing the levels of other lysosomal proteases. These results demonstrate that at late stages of VV infection, reductions in cellular Ii levels coupled with changes in lysosomal protease activity, contribute in part to defects in class II presentation.

Keywords

antigen presentation; viral; B cell

Introduction

VV is a complex DNA virus that replicates in the host cell cytoplasm. VV has been used as an attenuated vaccine to prevent smallpox transmission and has been proposed as a vaccine for infectious agents and tumors. While VV is highly effective in inducing long-lasting protective immunity in a majority of healthy individuals, potentially serious complications following VV infection have limited enthusiasm for this virus as a universal vaccine reagent (1–3). VV evades

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the host immune system affecting both innate and adaptive immunity (4). In epithelial and fibroblast cells, VV decreases host protein synthesis by disruption of host DNA replication (5), interference with cellular RNA synthesis and processing, as well as promoting RNA degradation (6,7). Yet, not all host gene expression is blocked with VV infection, as the virus exploits host proteins to survive and replicate. A recent analysis of dendritic cell (DC) gene expression upon exposure to attenuated VV, suggests altered levels of some co-factors for antigen presentation pathways (8).

Studies have demonstrated diminished MHC-mediated Ag presentation upon APC infection by VV (9–19). An early, transient reduction in $CD4^+$ T cell responses after VV vaccination of humans, suggests diminished APC function *in vivo* following exposure to VV (18). Minimal to no virus infection of activated and resting T cells has been reported (20,21). By contrast, APC (monocytes, DC and B cells) are highly susceptible to VV infection (11,21,22). VV disruption of MHC class II-restricted Ag presentation is observed with both professional and non-professional APC(10,13–15). While inhibition of host protein synthesis was postulated as one potential explanation for virus inhibition of class II presentation (13), others demonstrated sustained surface expression of class II proteins in APC up to 24 h after VV exposure (10, 11).

MHC class II complexes consist of αβ dimers which pair with the chaperone protein Ii in the ER. These αβIi complexes transit to the Golgi and are targeted to endosomes where Ii is degraded by acidic proteases such as cathepsins (Cat) S and L (23,24). HLA-DM releases Ii fragments from the ligand binding groove of class II to permit binding of peptide fragments from processed Ags. Finally, these peptide-class II complexes transit to the surface of APC for presentation to $CD4^+$ T cells. As a chaperone, Ii directs the folding of the class II αβ dimer, and transports class II molecules to compartments rich in antigenic peptides. Thus, Ii influences class II protein function and the selection of $CD4^+$ T cells (25–29).

In this report, significant reductions in intracellular Ii protein and mRNA levels were detected in human B-lymphoblastoid cell lines (B-LCL) at the late stages of VV infection. Yet even late in infection, low levels of new class II protein synthesis could readily be detected. Like VV, CHX also decreased Ii protein expression and class II function, while surface class II expression was maintained. Proteolytic processing of Ii was observed in both VV and CHX treated APC, contributing to diminished intracellular Ii. Yet, alterations in lysosomal protease expression were apparent only in VV infected APC. Virus infection induced host cathepsin L expression while diminishing the levels of several other lysosomal proteases. Thus, at late stages of B cell VV infection, reductions in intracellular Ii likely contribute to reduced APC function.

Materials and Methods

Virus and cells

VV, Western Reserve strain was cultured, gradient purified, and titered as previously described in CV-1, African green monkey kidney cells (10). CV-1 cells were cultured in DMEM (Invitrogen Life Technologies) with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Priess, a human B-LCL expressing class II DR4 and PriessGAD, Priess cells retrovirally transduced for constitutive expression of human glutamic acid decarboxylase (GAD), were cultured in IMDM (Invitrogen Life Technologies) with 10% heat-inactivated calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. T cell hybridomas, 33.1 specific for $GAD_{273–285}$, and 17.9 specific for human serum albumin $(HSA)_{64–72}$ both presented in the context of HLA-DR4, were cultured in RPMI 1640 (Invitrogen Life Technologies) with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM beta-mercaptoethanol (2-ME). HT-2, an IL-2 dependent T cell line was grown in RPMI 1640 media with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME and 20% T stim (BD

Biosciences). For quantification of IL-2 using HT-2 cells, T stim was omitted from the culture media.

Peptide and protein Ags

Peptides were manufactured using F-moc technology with HPLC analysis confirming purity >90% (10,30). HSA protein (protease free preparation) was obtained from Sigma.

Ag presentation assays

APC were cultured in the absence or presence of VV (MOI=10) for 2–14 h. APC were then washed, and fixed with 0.5% paraformaldehyde for 10 min at 4°C, followed by co-culture with T cell hybridomas for 24 h. VV infection was observed in >90% of these B cells as detected by viral Ag expression (10). To monitor the effect of virus replication on endogenous GAD presentation, PriessGAD were treated with VV +/− the viral DNA polymerase inhibitor, arabinosylcytosine (Ara C, 10 μM from Sigma) for 0–14 h before fixation and T cell addition. To investigate the effect of CHX on endogenous GAD Ag presentation, PriessGAD were treated with 10 μg/ml CHX (Sigma) for 0–6 h before fixing and co-culturing with GAD-specific T cells. To compare the effects of CHX and VV on exogenous Ag presentation, APC were treated with 1 μM HSA Ag +/− 10 μg/ml CHX for 6 h or VV (MOI=10) for 14 h, then fixed and co-cultured with HSA-specific T cells. To determine the effects of CHX or VV on peptide presentation, Priess cells were co-incubated with 1 μM $HSA_{64–76}$ or $GAD_{273–285}$ peptide +/− CHX or VV before addition of T cells. APC viability was assessed by trypan blue exclusion during exposure to virus or CHX, and conditions optimized to ensure little to no decrease in cell viability during these treatments. T cell activation was quantified by IL-2 production using an HT-2 bioassay (31). All assays were performed in triplicate, and the relative mean proliferation and standard deviations expressed as cpm.

Western blotting

Cells treated with VV (MOI = 10) +/− an optimal concentration of Ara C (10 μM) or with CHX (10 μg/ml) alone for different times, were harvested and lysed in buffer (10mM Trizma Base, 150mM NaCl and 1% Triton-X 100 with 1% protease inhibitors N^{α}-tosyl-lysine-chloromethylketone and phenylmethylsulphonyl fluoride). Cell lysates were centrifuged to remove nuclei prior to the addition of SDS sample buffer. Samples of 40 μg or 400 μg (the latter for cathepsin analyses only) of total cell protein were fractionated by SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were probed for: DRα monomer expression using the monoclonal antibody (mAb) DA6.147; DRαβ dimer using mAb L243; MHC I heavy chain using mAb 3B10.7; GAD using a polyclonal Ab (Sigma); Ii using mAb Pin 1.1; Cat S using Cat S Ab (Biovision); and Cat L using the mAb CPLH 36.1 (32). Cellular actin was detected using mAb-Pan Actin Ab-5 (Lab Vision), and GAPDH using GAPDH mAb (Millipore). VV Ags were detected using a rabbit antisera recognizing abundant virion Ags (Cortex Biochem), as well as rabbit antibodies specific for epitopes within the D8 (32 KDa) and H3 (35 KDa) late viral Ags. Viron Ags D8 and H3 were similarly detected using these Abs. Densitometric analysis of Western blots was carried out with software ImageJ (NIH website) using cellular actin/GAPDH expression as a relative internal standard.

To monitor the effects of VV on Ii maturation and processing, PriessGAD cells were cultured with VV (MOI=10) for 2 h to permit infection, followed by incubation with protease inhibitors for an additional 12 h for a total infection time of 14 h before analysis by Western blotting. Inhibitors used here were 0.5 mM leupeptin (Leu, Sigma); 0.5 mM E64 (Sigma); and 1 mg/ml AEP inhibitory peptide AENK (33,34). To study the effects of CHX on Ii processing, PriessGAD cells were pretreated with Leu for 1h, then CHX (10 μg/ml) was added to the media for an additional 4 h followed by Western blotting and densitometry. Cell viability was >80% after each inhibitor treatment.

[^{35}-S] methionine incorporation and pulse-chase assays

To assess the effects of VV on host protein synthesis, PriessGAD cells (1×10^7/sample) were infected with VV (MOI=10) for 0, 2, 6, and 14 h before addition of 0.5 mCi of ^{35}S-methionine (MP Biomedicals) in cold methionine free RPMI media for 1 h. Cells were harvested, lysed and immunoprecipitated with mAb Pin 1.1 for Ii and DA6.147 for DR. The precipitated samples were resolved on the SDS-PAGE followed by Coomassie Blue gel staining and autoradiography. Coomassie Blue gel staining confirmed equal sample loading. For pulse-chase assays, PriessGAD cells were starved in methionine free RPMI media for 1 h and labeled with ^{35}S-methionine for one hour. Samples were chased in media with unlabeled methionine +/− VV (MOI=10) and/or E64 (500 μM) for 0, 2, 6, and 14 h. Samples were immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

Semi-quantitative RT-PCR and quantitative real time PCR

PriessGAD cells were treated +/− VV (MOI = 10) for up to 14 h. Total cellular RNA was extracted with an RNEasy Mini kit (Qiagen). cDNA was generated from RNA using an Advantage RT for PCR kit (BD Biosciences). Primers for PCR amplification were designed using the Custom Primers - OligoPerfect™ Designer software (Invitrogen). The primers used for human Ii were 5′-GCT GTC GGG AAG ATC AGA AG-3′ (sense) and 5′-GCC ATA CTT GGT GGC ATT CT-3′ (antisense); for DRα were 5′-CAA AGA AGG AGA CGG TCT GG-3′ (sense) and 5′-AGC ATC AAA CTC CCA GTG CT-3′ (antisense); for Cat S were 5′-GGA TCA CCA CTG GCA TCT CT-3′ (sense) and 5′-CCA GCT TTC CTG TTT TCA GC-3′ (antisense); for Cat B were 5′-GCT ATC CTG CTG AAG CTT GG-3′ (sense) and 5′-CAT TGT CAC CCC AGT CAG TG-3′ (antisense); for Cat D were 5′-AGC TGG TGG ACC AGA ACA TC-3′ (sense) and 5′-CTC TGG GGA CAG CTT GTA GC-3′ (antisense); for Cat L were 5′-TGT GGT TCT TGT TGG GCT TT-3′ (sense) and 5′-CAG GCC TCC ATT ATC CTG AA-3′ (antisense); for actin were 5′-AGA AAA TCT GGC ACC ACA CC-3′ (sense) and 5′-CCA TCT CTT GCT CGA AGT CC-3′ (antisense); for E3L were 5′-CGC AGA GAT TGT GTG TGA GG-3′ (sense) and 5′-AAC GGT GAC AGG GTT AGC AC-3′ (antisense); for D8L were 5′-CAA ATC GGA CAA CCA TCT CA-3′ (sense) and 5′-CCA TTA GAT CCG CCA ATA CG-3′ (antisense); and for HSC70 were 5′-AGC TGT AAG ACG CCT CCG TA -3′ (sense) and 5′-GTG ACA TCC AAG AGC AGC AA -3′ (antisense). GAPDH primers were obtained from the Advantage RT for PCR kit. Amplification reactions were performed using 1.1×ReddyMix™ PCR Master Mix (ABgene) with different cycle times in a MJ Research thermal cycler. The number of amplification cycles for semi-quantitative analysis was 28 cycles except for HSC70, Cat S, and Cat D (32 cycles); Cat L (40 cycles); and Cat B (60 cycles). The cycling parameters used were: 95°C, 15 sec; 50°C, 30 sec; and 68°C, 1 min. PCR products were electrophoresed on 1.5% agarose gels, stained with SYBR® safe DNA gel stain (Invitrogen), and detected with UV transillumination using ChemiDoc™ XRS (Bio-Rad). mRNAs for early viral gene product E3L and late gene product D8L mRNAs were monitored as evidence of VV infection. Host cell HSC70 was used as a control for sample loading. Similar procedures were used to detect relative mRNA abundance after CHX treatment. Ii mRNA expression levels in CHX treated samples were evaluated and averaged from 3 independent experiments with the standard deviation indicated.

To detect Ii, DRα and HSC70 mRNA levels by quantitative real time PCR, RNA isolation and cDNA synthesis were performed as above. TaqMan® Gene Expression Assays with specific primers and probes (Hs00269961_m1 for Ii; Hs00219575_m1 for DRα and Hs00852842_gH for HSC70) were incubated with TaqMan® Fast Universal PCR Master Mix and cDNA templates. Samples were amplified 40 cycles using a 7500 Real-Time PCR System (Applied Biosystems) with the following parameters: 95°C, 15 sec; and 60°C, 1 min. HSC70 was used as the endogenous reference while 0 h relative quantification of Ii or DRα mRNA was used as the calibrator.

Flow cytometry

For surface CLIP staining, cells were incubated with FITC-anti-human CLIP Ab or corresponding isotype control Ab (BD Biosciences) on ice for 30 m followed by washing and fixation with 0.5% paraformaldehyde. Samples were gated on live cells and analyzed by flow cytometry using Cell Quest software (BD Biosciences).

DNA sequencing

To confirm the PCR product amplified using primers specific for human Cat L, PriessGAD cells were cultured +/− VV (MOI = 10) for 14 h followed by mRNA isolation and cDNA synthesis. After amplification of cellular mRNA using the Cat L primers, the reaction products from each sample were separated on a 1% agarose gel, and the amplified cDNA bands were excised and extracted using a QIAquick gel extraction kit (Qiagen). The cDNAs were sequenced by the DNA Sequencing Core Facility at IUSM using a Perkin Elmer/Applied Biosystems 3100 Genetic Analyzer and Big Dye Terminator chemistry v3.1. Cat L cDNA amplified from uninfected PriessGAD cells was sequenced and compared with this gene amplified from VV infected PriessGAD cells using ClustalW software from the European Bioinformatics Institute. To confirm the identity of these amplified cDNA sequences as human Cat L, nucleotide-nucleotide BLAST software from the National Center for Biotechnology Information was used.

Results

MHC class II function and protein expression during VV infection of APC

VV inhibits MHC class II-mediated presentation of Ags and peptides by APC (10). Viral infection of PriessGAD cells reduced MHC class II presentation of an endogenous Ag, GAD dependent on viral MOI and the duration of infection (Fig. 1*A* and *B*). The inhibitory effects associated with virus infection progressed over time without diminishing cell viability.

The inhibitory effect of VV on Ag presentation was observed initially at early stages of APC infection (≤ 2 h), and prior studies demonstrated this effect was independent of VV replication (10). VV blocks host protein synthesis shortly after entry into fibroblasts (35). Thus, it remained possible that VV disruption of Ag presentation was linked to changes in the expression of MHC class II proteins or other components of class II pathway during virus infection. Protein levels for both MHC class I and class II molecules were assessed during 24 h of VV infection. VV infection did not significantly diminish steady state expression of MHC class II DRα monomers or DRαβ dimer levels up to 24 h (Fig. 1*C* and *D*). Also, VV infection of B-LCL did not influence the level of endogenous GAD Ag, MHC class I heavy chain and cellular actin expression in B-LCL. As expected, the expression of viral late membrane D8 and H3 antigens increased as infection progressed.

VV infection decreased Ii expression in B cells at both the protein and mRNA levels

Although expression of MHC class II proteins remained constant, steady state levels of cellular Ii decreased in a time dependent manner upon VV infection of B-LCL (Fig. 2*A*). Ii protein levels were slightly decreased at 2 h of infection with a more pronounced drop between 8–12 h of infection. Densitometric quantification revealed a 15% reduction in cellular Ii abundance at 2 h, with an 80% decrease in Ii levels at 12–14 h of VV infection. To determine if the reduction in Ii expression occurred at the level of protein synthesis, biosynthetic radiolabeling was performed. PriessGAD cells were infected with VV for up to 14 h with radiolabeled methionine added at different times prior to immunoprecipitation of Ii and class II proteins. At 6 h, VV infection decreased Ii synthesis by 44% yet DR synthesis was preserved (Fig. 2*B*). Even at 14 h, new synthesis of class II was detected but little if any new Ii protein was

synthesized by infected cells. To further establish that VV inhibits Ii synthesis, the relative abundance of Ii mRNA was measured using semi-quantitative RT-PCR analysis. Ii mRNA levels progressively decreased after VV infection, temporally preceding the drop in Ii protein expression (Fig. 2*C*). Host heat shock cognate protein 70 (HSC70) mRNA levels, used as a loading control, did not change up to 14 h after VV infection. To more accurately monitor alterations in Ii mRNA levels after VV infection, real time PCR was performed using HSC70 as the endogenous reference. Quantification of Ii mRNA during VV infection revealed a 65% decrease after 6 h and an 80% drop after 10 h (Fig. 2*D*). These results indicate diminished Ii synthesis contributes to the reduced steady state levels of Ii protein during VV infection. To further investigate the effect of virus on Ii expression and GAD presentation, B-LCL were treated with VV +/− arabinosylcytosine (AraC) for 14 h. AraC is a virus-specific DNA polymerase inhibitor which blocks replication and translation of late viral gene transcripts (36). As shown in Fig. 2*E*, decreased Ii expression and GAD presentation were observed at 14 h of VV infection, but could be partially prevented by AraC treatment. AraC alone did not affect cellular Ii expression or GAD presentation. AraC treatment did not alter the effects of virus on Ag presentation at 6 h. Similar results were obtained at higher concentrations of AraC. Expression of viral late Ags D8 and H3 were reduced in cells exposed to VV and AraC. Studies using UV treated virus also indicated virus replication was not essential for the observed loss of class II function (10). Thus, studies with AraC suggest at late stages of virus infection, loss of cellular Ii contributes in part to decreased class II function.

Inhibition of host protein synthesis with CHX rapidly reduced cellular Ii levels with no immediate change in MHC class II steady state protein expression

CHX, a protein synthesis inhibitor, has been used to investigate the effects of disrupting host protein synthesis on the class II pathway as well as to study changes in host protein expression with poxvirus infection (29,37). CHX interrupts both the initiation and extension of de novo protein synthesis by acting at the translation level (38,39). Here, CHX efficiently decreased B cell protein synthesis with a minimal loss of cell viability after 6 h, as determined by monitoring radiolabeled amino acid incorporation into host proteins (data not shown). Cell lysates from CHX treated B-LCL were assessed for class II DRα, DRαβ, class I, GAD and Ii protein expression (Fig. 3*A*–*C*). Similar to VV infection, Ii protein levels were significantly diminished with CHX treatment, yet steady state levels of other class II pathway components and endogenous GAD Ag expression were relatively unaffected. CHX rapidly diminished cellular Ii protein expression in a time dependent manner, and this loss of Ii was significantly faster than observed with VV (Fig. 3*C* and 2*A*). At longer times of treatment, CHX induced cytopathic effects hence studies were limited to 6 h. Consistent with CHX acting at the translational level, mRNA for both Ii and DRα remained constant after CHX treatment of B-LCL for 6 h (Fig. 3*D*).

Inhibition of MHC class II presentation by CHX treatment of B-LCL

In contrast with VV, published studies had demonstrated reductions in class II Ag but not peptide presentation upon B cell exposure to CHX (29,40). Studies were performed to determine whether similar changes in class II function were observed using CHX concentrations which promote the loss of cellular Ii. As shown in Fig. 4*A*, CHX treatment inhibited endogenous GAD Ag presentation in a time dependent manner. The reduction in endogenous GAD Ag presentation correlated temporally with the loss of Ii protein in CHX treated B-LCL (Fig. 3*C*). As shown in Fig. 4*B*, exposure of B-LCL to CHX significantly reduced exogenous Ag presentation comparable to VV inhibition. The effect of CHX or VV on peptide presentation by B cells was tested (Fig. 4*C* and *D*). CHX treatment of B-LCL inhibited HSA peptide presentation much like VV infection (Fig. 4*C*). However, CHX consistently disrupted GAD peptide presentation less severely than VV infection (Fig. 4*D*). HSA peptide must be processed by APC in early endosomes before presentation by class II

molecules (30). By contrast, the GAD epitope can directly bind to surface class II without processing (41). Previous studies also suggested minimal inhibition of surface peptide presentation by CHX (29,40). Thus, while both CHX and VV disrupt class II Ag presentation by B cells, differences in the ability of each agent to perturb peptide presentation were observed. Class II presentation of protein Ags was typically more sensitive to inhibition by VV or CHX, when compared with class II display of exogenously added peptides and these agents.

VV infection influences Ii degradation

Although protein synthesis inhibition is likely the predominant mechanism leading to diminished Ii protein expression in cells exposed to VV or CHX, the proteolytic cleavage of this chaperone within endosomal compartments may also influence Ii steady state abundance. Within these organelles, Ii is proteolytically cleaved to leupeptin-induced peptide (LIP), small LIP (SLIP) and then CLIP, a terminal product of Ii degradation. Disruption of Ii proteolysis can perturb class II maturation and function. It has been established that some viral proteins, such as human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus virion-associated protein Vpx, reduce cellular Ii expression by promoting Ii degradation (42).

To test if VV infection alters Ii degradation, B-LCL were exposed to VV in the presence of leupeptin (Leu). Leu inhibits cysteine proteases such as Cat B, L, S reversibly, perturbing Ii degradation and resulting in cellular Ii and LIP accumulation (34,43). PriessGAD cells were pretreated +/− VV, followed by culture with Leu and analysis of cellular Ii levels by Western blotting. In Fig. 5*A*, VV infection decreased cellular Ii levels by 79% compared to controls at 14 h. Leu treatment of uninfected APC (1×Leu) resulted in Ii and LIP accumulation. Cellular VV infection coupled with Leu treatment decreased Ii levels by about 60%, compared with Leu treatment only. Similar levels of viral infection were found in cells +/− Leu as assessed by the relative abundance of VV proteins. These results indicate Ii proteolysis continued during VV infection, coupled with diminished Ii synthesis. Studies suggest asparagine endopeptidase (AEP) can catalyze the earliest steps in Ii proteolysis (33). The addition of an AEP inhibitor AENK to APC resulted in Ii accumulation, consistent with a role for AEP in Ii proteolysis. AENK treatment did not alter virus infection, but similar to Leu preserved Ii levels in cells, consistent with AEP functioning in Ii proteolysis during VV infection. Analysis of the later steps in Ii processing revealed low amounts of LIP at 14h in VV + Leu treated cells compared with uninfected cells plus Leu (Fig. 5*A* and *C*). To suppress cellular Ii proteolysis during VV infection, repeated additions of Leu or an irreversible cysteine protease inhibitor, E64 were required, leading to greater LIP and Ii accumulation (Fig. 5 *A* and *C*). These results suggested enhanced Ii processing at the late stages of VV infection. Consistent with this result, flow cytometry revealed slightly elevated levels of the Ii fragment CLIP at the late stages of APC VV infection (Fig. 5*E*). Thus, AEP and Leu-sensitive proteases appear to play important roles in Ii processing during VV infection. In testing if inhibition of cellular protein synthesis directly perturbs Ii processing, CHX plus Leu treatment of APC minimally altered Ii steady state levels compared to CHX treated cells (Fig. 5*B*). The ratio of LIP/Ii accumulation also was not significantly altered in cells treated with CHX and Leu vs. Leu alone (Fig. 5*C*). Thus, Ii processing was unchanged in CHX treated cells. The kinetics of Ii degradation were monitored using biosynthetic radiolabeling and pulse-chase analysis. PriessGAD cells were pulse labeled with [^{35}S-] methionine for 1 h, then the chase initiated +/− VV and/or E64 for 0–14 h. As shown in Fig. 5*D*, the amount of radiolabeled Ii decreased progressively in control cells between 0–14 h due to proteolytic processing. Cellular VV infection appeared to slow Ii degradation at early times, i.e. 6 h as suggested by the relative abundance of Ii compared to control cells. Consistent with this, slightly less LIP was seen in VV infected cells exposed to E64 at 6 h. At later stages of infection (14 h), enhanced Ii degradation was apparent as reflected by the decrease in radiolabeled Ii in VV vs. control cells. Thus, virus-induced alterations in Ii degradation were detected during infection.

VV altered cellular lysosomal protease expression at both the mRNA and protein levels

Although the steady state expression of MHC class II proteins was not altered, analysis of mRNA for class II and other co-factors of Ag processing revealed differential expression with VV infection (Fig. 6*A*). As shown in Fig. 6*A*, viral E3L message was detected early during infection, while late gene D8L message progressively increased. Host MHC class II DRα as well as Cat S, Cat B and Cat D mRNA levels were progressively reduced during VV infection. Diminished DRα mRNA levels after VV infection were confirmed by real time PCR (data not shown). Acidic cathepsins are important for processing both Ii and Ag (34,44). Among the cathepsins tested, Cat S plays a key role in the later stages of Ii cleavage in B cells (24,45). Thus, a reduction in Cat S mRNA may lead to aberrant Ii processing. Viral infection also led to a decrease in message for Cat B and D. These cathepsins function in processing Ag within APC (44,46). Notably, Cat L mRNA increased in a time dependent manner during VV infection of B cells (Fig. 6*A*). Cat L mediates Ii processing in cortical thymic epithelial cells to regulate positive selection (23,47). A recent study demonstrated Cat L expression in human B cell lines (24,48–53). To further confirm Cat L expression in B cells +/− VV infection, the detected mRNA was amplified, converted to cDNA, and sequenced. The more abundant amplified sequence from VV infected cells as well as the cDNA sequenced in control cells were both identified as human Cat L. To test whether altered cathepsin expression was observed during VV infection, Western blotting analysis was carried out with cathepsin-specific Abs. Corresponding with mRNA levels, VV infection of APC reduced Cat S protein abundance while enhancing Cat L protein levels (Fig. 6*B*). Reduced Cat S levels may account for reduced or slow Ii degradation at the transition from early to late stages of VV infection. Later, induction of cellular Cat L likely substitutes for Cat S to promote enhanced Ii proteolysis in virus infected B cells (Fig. 5*C*). By contrast, CHX treatment did not alter the abundance of these two cathepsins in APC after short incubations.

Discussion

Although the disruption of MHC class II Ag presentation by VV infection has been well established (10), the precise events leading to this inhibition remain poorly defined. Multiple steps within the class II pathway may be perturbed by VV during the transition from early to late stages of infection. Yet few studies have addressed temporal changes within APC during VV infection that might influence class II presentation. VV infection of APC reduces peptide binding to class II molecules, suggesting destabilization of class II and consequently reduced presentation to $CD4^+$ T cells (10). In the current study, a time dependent loss of Ii protein and mRNA was observed during the late stages of VV infection of human B cell lines (Fig. 2). Ii is important for class II αβ maturation and the presentation of endogenous and exogenous Ag as well as peptides. While the virus-induced loss of Ii was gradual, by 14 h of infection little if any new Ii synthesis was detected (Fig. 2). Yet, new synthesis of class II subunits could still be observed at this time. Immunoblotting also revealed a significant depletion of the total pool of cellular Ii at this late stage. Similar substantial losses in cellular Ii induced either by antisense oligodeoxynucleotides or gene deletion reduced class II presentation of select Ags by 60–90%, while enhancement of Ii expression by either transfection or knock-in increased Ag presentation (25,26). Thus, reductions in cellular Ii during VV infection likely contribute to the loss of class II function.

To study the mechanisms responsible for Ii loss during APC infection by VV, two pathways were explored: inhibition of Ii synthesis and enhanced Ii degradation. Expression of Ii mRNA and new protein synthesis decreased during infection, contributing to the decrease in steady state cellular Ii protein between 6–14 h (Fig. 2). A similar reduction in Ii mRNA was observed upon VV infection of human PBMC (data not shown). In B cells, these changes were observed during the late stages of VV infection and paralleled viral late protein expression (Fig 1, 2 and

6). Class II mRNA levels also decreased at this stage of infection, yet new synthesis of class II subunits persisted at low levels at 14 h and surface class II protein expression was stable up to 24 h. Class II complexes in human B cells display half-lives of about 36 h, likely accounting for the preservation of surface class II (54). Aggregation of some murine class II alleles is observed in Ii-deficient mice (28). Yet during VV infection, such high molecular mass aggregates of class II DR4 were not observed. Studies with Ii-deficient mice also suggest DM expression is reduced (28,55). However, no changes in DM levels were found in human B cell lines infected with VV (data not shown). Thus, virus-induced changes in the class II pathway in these human B cells do not precisely mirror the effects of Ii loss observed with some Ii-deficient mouse strains (28,55). Analysis of intracellular αβIi complexes during VV infection of B cells did reveal reduced levels of Ii co-precipitating with αβ after 10–12 h (data not shown). Reduced levels of αβIi complexes were also detected in murine cell treated with CHX (29). In the current study, CHX treatment of APC rapidly led to reduced cellular Ii expression and defects in both Ag and peptide presentation by human APC.

The effects of VV infection on the class II pathway go beyond altering host protein synthesis, as changes in cathepsin expression were also detected during virus infection. Expression of Cat S, which is required for terminal Ii proteolysis was greatly diminished during virus infection. Pulse-chase studies revealed Ii proteolysis was initially slowed during VV infection (Fig. 5*C*), consistent with an observed decrease in Cat S levels. The half-life of Ii in human B cell lines is typically 2–2.5h (34,56). Yet late in VV infection, Ii degradation appeared to be enhanced, possibly due to changes in the cell's protease content. VV infection of human B cells resulted in induction of Cat L mRNA and increased expression of the active form of Cat L protease. Remarkably, mRNA expression of other lysosomal cathepsins, Cat S, B and D decreased with virus infection, suggesting cellular proteolytic processing is altered. Cat S controls LIP processing in many APC. Yet in thymic epithelial cells Cat L substitutes for Cat S to proteolyze LIP and promote class II maturation. Why VV infection promotes increased Cat L expression is unclear. Viral induction of host Cat L may facilitate infection, as has been observed for mouse hepatitis virus type 2 and acute respiratory syndrome coronavirus (57, 58). Or VV could use Cat L to promote extracellular matrix degradation like tumors (59,60). Alternatively, elevated Cat L may represent a stress response by the host during infection to possibly accelerate processes such as apoptosis (61,62). The mechanisms by which cellular Cat L are up-regulated remained to be elucidated. Little is known about specific features of this gene's promoter that might be modulated by VV, but studies do suggest that stress can up-regulate Cat L expression at the promoter level (63,64). Notably in this study, inhibition of host protein synthesis using CHX failed to alter cellular Cat S or Cat L protein levels at short times of exposure. Whether VV-induced changes in cathepsin expression influence Ag processing has not been determined. Yet consistent with this possibility, the peptide content of class II was altered during cellular infection with an attenuated VV, MVA (65). Changes in the type of peptides as well as their terminal residues were found upon comparing class II DR ligands from MVA and uninfected cells. Inhibition of class II presentation of Ags and peptides was detected within 1–2 h of VV infection (10). Studies suggest a destabilization of class II at this early stage of infection, as peptide binding was altered. Disruption of Ii expression and changes in Ii processing were temporally observed later during the virus life cycle. Together these results suggest VV may disrupt the class II presentation pathway by multiple mechanisms including perturbing the class II chaperone Ii. These results have implications in terms of our understanding of both VV pathogenesis and host immune responses to poxvirus infection.

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Abbreviations used in this paper

AEP	Asparagine endopeptidase
AraC	arabinosylcytosine
B-LCL	B lymphoblastoid cell line
Cat	cathepsin
CHX	cycloheximide
DC	dendritic cell
GAD	glutamate decarboxylase
HSA	human serum albumin
HSC	heat shock cognate protein
Ii	invariant chain
Leu	leupeptin
LIP	leupeptin-induced peptide
MOI	multiplicity of infection
SLIP	small LIP
and VV	vaccinia virus

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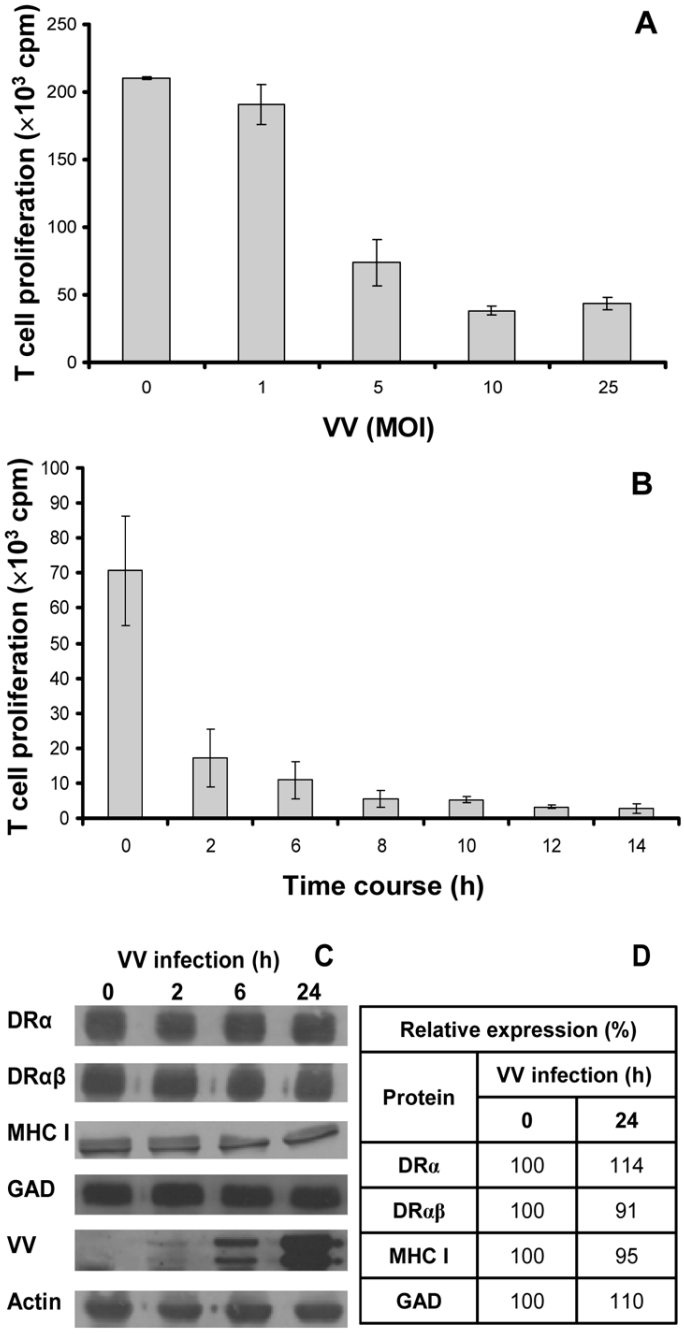

Relative expression (%)		
Protein	VV infection (h)	
	0	24
DRα	100	114
DRαβ	100	91
MHC I	100	95
GAD	100	110

Fig. 1. Maintenance of MHC class II protein expression during VV inhibition of Ag presentation

A. Inhibition of class II presentation by VV was dependent on viral MOI. PriessGAD cells were infected with VV (MOI 0 to 25) for 6 h, prior to analysis of GAD Ag presentation. *B.* Temporal progression of VV inhibition on class II function. PriessGAD cells were incubated with VV (MOI=10) 0 to 14 h before analysis of Ag presentation. *C.* Steady state MHC class I and class II expression was maintained during VV infection. PriessGAD cells were infected with VV (MOI=10) and samples immunoblotted for protein expression including the presence of VV Ags D8 and H3.. *D.* Densitometric analysis of protein expression. For samples in panel *C*, each protein's abundance at 0 and 24 h was calculated relative to actin. 24 h samples were

normalized to 0 h samples which had been set as 100%. Data are representative of at least 3 independent experiments.

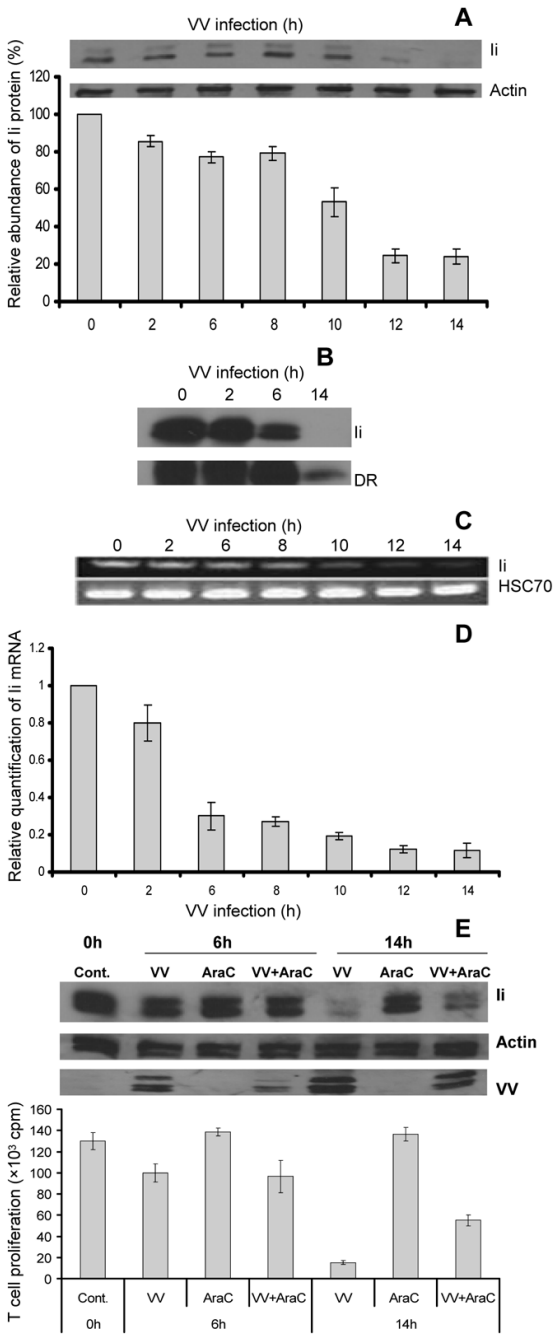

Fig. 2. VV infection reduced Ii expression at both the protein and mRNA levels

A. VV reduced Ii steady state protein levels during APC infection. Samples of PriessGAD cells infected with VV for up to 14 h were Western blotted for Ii expression. Actin was used as the loading control. Densitometric quantitation of Ii protein expression was calculated relative to actin abundance at each time point after VV infection of B cells (averaged from 3 experiments). *B.* VV infection decreased new Ii protein synthesis more rapidly than class II protein levels. PriessGAD cells were cultured with ^{35}S-methionine for 1 h at times 0, 2, 6 or 14 h of incubation with infectious VV. Cell lysates were immunoprecipitated with Ii or class II specific Abs and precipitated proteins were resolved by SDS-PAGE and autoradiography. *C.* VV infection reduced B cell Ii mRNA levels in a time dependent manner. PriessGAD cells were infected

with VV (MOI=10), mRNA levels for Ii and HSC70 were analyzed with RT-PCR. The relative abundance of HSC70 was tracked as the loading control. *D.* Quantitative real time PCR analysis of Ii mRNA during VV infection. mRNA was isolated from PriessGAD cells infected with VV and amplified with Ii and HSC70 specific primers. Quantification of Ii mRNA levels was determined relative to HSC70 message levels. The results of 3 experiments were averaged. *E.* Inhibition of viral DNA replication and late protein synthesis partially preserved Ii expression and class II function. PriessGAD cells were treated with VV +/− the viral DNA polymerase inhibitor-arabinosylcytosine (AraC, 10 μM) for 0, 6 and 14h. Western blot analysis and T cell assays were performed to monitor Ii, actin and VV late Ag D8 and H3 expression as well as GAD Ag presentation.

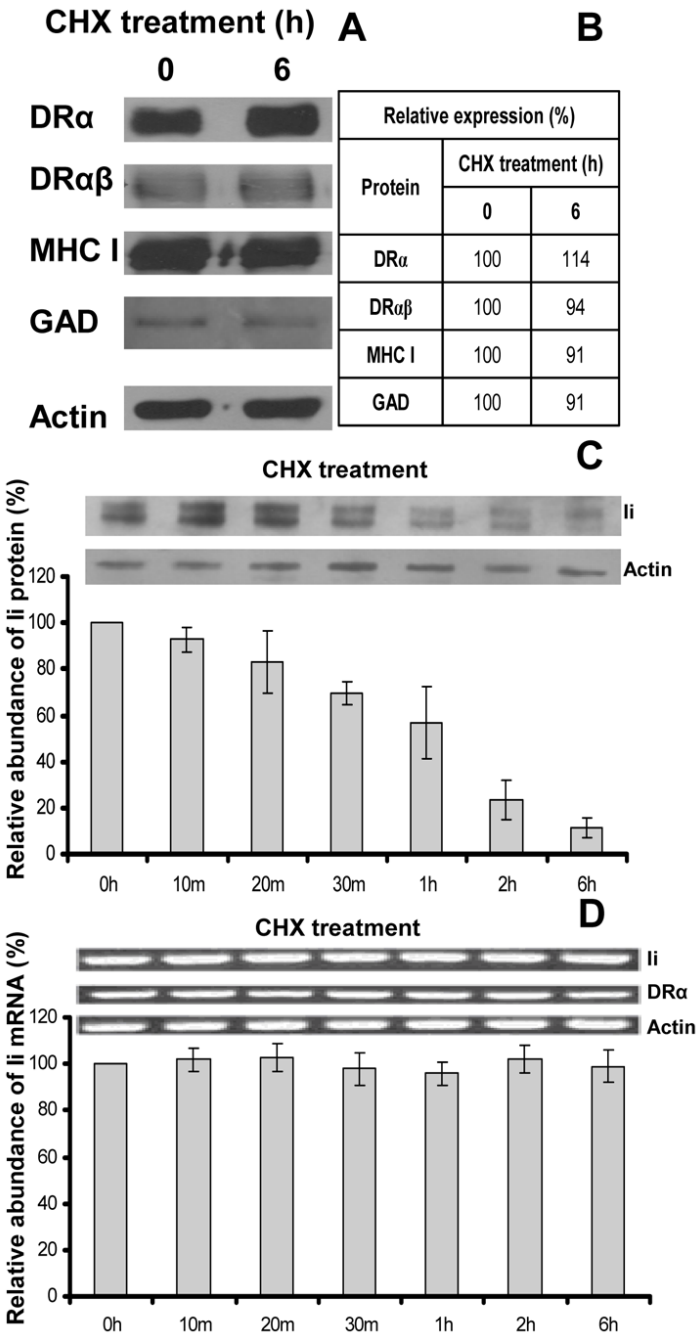

Fig. 3. CHX treatment of B cells reduced Ii protein but not mRNA levels

A. Class II protein remained constant after CHX treatment. PriessGAD cells were treated with CHX (10 μg/ml) and lysed for Western blot analysis. Actin protein expression was used as a control for sample loading. *B.* Densitometric analysis of protein expression. Protein abundance at 6 h plus CHX was compared with 0 h samples using actin as the endogenous control. *C.* CHX treatment of APC rapidly reduced Ii protein levels. PriessGAD cells were treated with CHX (10 μg/ml), followed by Western blot analysis. The average abundance of Ii protein relative to actin after CHX treatment from 3 independent studies is shown. *D.* CHX treatment of B cells did not change Ii mRNA levels. PriessGAD cells were treated with CHX (10 μg/ml)

for up to 6 h and analyzed by RT-PCR. Actin was used as the loading control. Quantification of Ii mRNA levels after CHX treatment (average of 3 RT-PCR experiments) is shown.

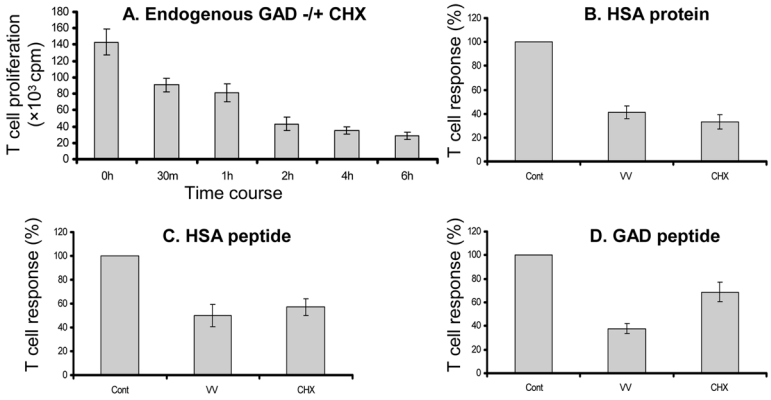

Fig. 4. Both VV and CHX treatment of human B cell lines decreased MHC class II presentation

A. The inhibitory effect of CHX on endogenous GAD Ag presentation. PriessGAD cells were incubated with 10 μg/ml CHX prior to analysis of Ag presentation. *B.* VV or CHX treatment of B cells inhibited exogenous HSA Ag presentation. Priess cells incubated with HSA Ag (1μM) were treated with VV (MOI=10) for 14 h or CHX for 6 h prior to assessing T cell activation. *C.* VV or CHX treatment of B cells inhibited HSA peptide presentation. Priess cells were incubated with HSA peptide (1μM) and VV for 14 h or CHX for 6 h before analysis of T cell activation. *D.* VV or CHX treatment of B cells inhibited GAD peptide presentation. Priess cells were incubated with GAD peptide (1μM) and VV or CHX before T cell activation evaluation. For Fig *B–D*, relative responses of T cell are shown for APC +/− treatment. Data are representations of at least 3 separate experiments.

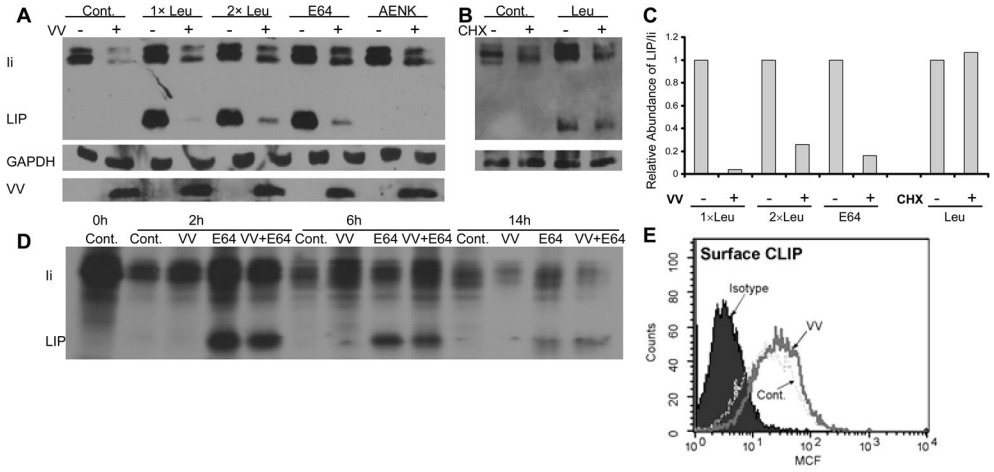

Fig. 5. Ii degradation in cells exposed to VV or CHX

A. Ii degradation in VV infected B cells. PriessGAD cells were incubated +/− VV (MOI=10) for 2 h, then Leu, E64 or AENK were added for an additional 12 h. For 2×Leu samples, an additional dose of Leu was added to cells after 8 h. Cells were harvested and analyzed by Western blotting. *B.* Ii processing in B cells exposed to CHX. PriessGAD cells were incubated with Leu for 1 h then CHX (10 μg/ml) was added for an additional 4 h followed by Western blotting. *C.* Relative abundance of LIP/Ii proteins in control and VV infected/CHX treated cells as in panels *A* and *B*. LIP and Ii levels in cells were determined using densitometry. *D.* Ii proteolysis in cells exposed to VV +/− E64. PriessGAD cells pulse-radiolabeled with ^{35}S-methionine were chase incubated in media +/− VV and/or E64 for 0, 2, 6, 14 h. Cell lysates were immunoprecipitated to capture Ii and LIP with Pin 1.1, and these proteins were analyzed by SDS-PAGE and autoradiography. *E.* Cell surface CLIP expression at late stages of VV infection. PriessGAD cells (control, dash line) or cells infected with VV (dark line) for 14 h were stained with a CLIP specific Ab and analyzed by flow cytometry. The shaded histogram represents isotype Ab staining of infected cells. Data are representative of at least 3 separate experiments.

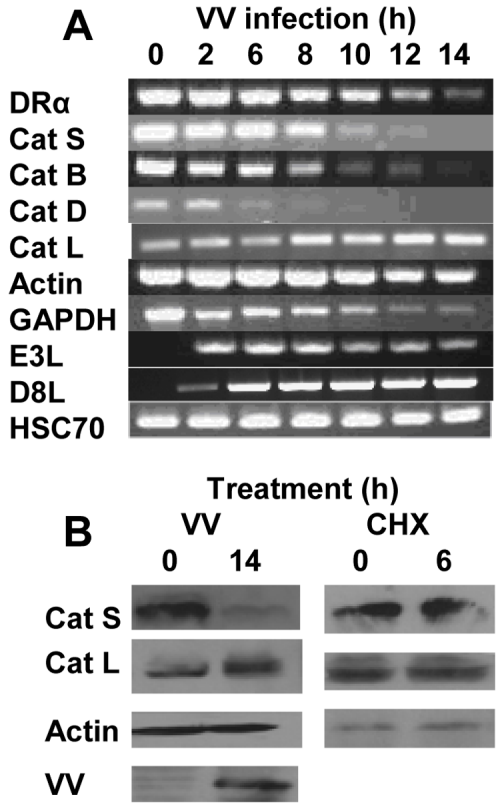

Fig. 6. VV infection altered mRNA and protein expression of cellular lysosomal proteases

A. Alteration in cellular mRNAs after VV infection. PriessGAD cells were infected with VV (MOI=10) and mRNA was isolated for RT-PCR. Viral E3L and D8L gene transcripts were monitored to confirm virus gene expression. *B.* Protein expression of host cathepsins after VV or CHX treatment. PriessGAD cells were treated with VV (MOI=10) (left column) or CHX (10 μg/ml) (right column) and harvested for analysis by Western blotting. VV proteins were detected to confirm viral infection. Actin expression levels were used as the sample loading control. Data are representative of at least 3 experiments.